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CLINICAL STUDY

Busuishengxue ranules mediate their effects upon non-severe aplastic anemia *via* mitogen-activated protein kinase/extracellular signal-regulated kinase pathway

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Abstract

OBJECTIVE: To observe the clinical efficacy of Busuishengxue granules on non-severe aplastic anemia (NSAA) and investigate its effect on the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway.

METHODS: Sixty NSAA patients were divided equally into two groups. Subjects in the experimental group were treated with Busuishengxue granules, and the control group with Zaizaoshengxue tablets. The treatment course was 6 months and curative efficacy was compared between the two groups as well as with 10 healthy individuals. Flow cytometry (FCM) was used to detect the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$). Western blotting was employed to detect the expression of enzymes in the MAPK/ERK pathway.

RESULTS: The efficacy of Busuishengxue granules was significantly better than that of Zaizaoshengxue tablets ($P<0.05$). Before treatment, expression of JNK, phospho-ERK 1/2 and p-JNK was higher, and $[Ca^{2+}]_i$ higher, than that of the control group ($P<0.05$). After treatment with Busuishengxue granules, expression of all enzymes related to signal transduction pathways in the blood cells of NSSA patients were altered to different degrees.

CONCLUSION: Busuishengxue granules had a better effect with regard to improving symptom scores, increasing the number of blood leukocytes, and increasing hemoglobin levels than Zaizaoshengxue tablets, and they differed slightly in terms of increasing the number of platelets.

Key words: Anemia, aplastic; MAP kinase signaling system; Busuishengxue granule; Zaizaoshengxue tablet

INDRODUCTION

Aplastic anemia (AA) is a disease in which the bone marrow (BM) is damaged. This causes a deficiency of red blood cells (RBCs), white blood cells (WBCs), and

platelets. AA can be classified as severe aplastic anemia and non-severe aplastic anemia (NSAA). The proliferation, differentiation and release of hematopoietic stem cells in the BM is achieved by the combination of hematopoietic stem-cell adhesion molecules, stromal cells, and adhesion proteins of the extracellular matrix.[1] Traditional Chinese Medicine (TCM) for AA has been used for many years. In patients with AA, TCM can be used to: regulate imbalances in the immune system; promote the proliferation and differentiation of stem cells; improve the hematopoietic microenvironment. As shown in our preliminary study, the abnormal adhesion and abnormal expression of integrins from the very late activation antigen (VLA) subfamily are closely related to NSAA. Abnormal expression of adhesion molecules such as VLA-2, VLA-4, VLA-5 and VLA-6 seemed to be implicated in NSAA. Levels of these adhesion molecules seem to differ before and after treatment with TCMs.[2-8] We inferred that abnormal expression of integrins from the VLA subfamily may be caused by abnormal expression of the intracellular signaling pathways mediated by them, especially the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway. Hence, we investigated the enzymes of the MAPK/ERK pathway as well as intracellular levels of the calcium ion ($[Ca^{2+}]_i$) to understand how treatment with TCM affects this pathway in patients with NSAA.

MATERIALS AND METHODS

Subjects

The study cohort was 60 subjects with NSAA. They were inpatients or outpatients from the Number One Hospital of the Heilongjiang University of Chinese Medicine (Heilongjiang, China) from November 2008 to April 2012. All subjects participated voluntarily in this study and provided written informed consent. They were divided randomly into the experimental group and control group. There were 30 patients (16 males and 14 females) in the experimental group and they were aged 14-67 (mean age, 36 ± 8) years. There were 30 cases (15 males and 15 females) aged 14-65 (mean age, 35 ± 8) years in the in the control group. Ten healthy volunteers (5 males and 5 females) with a mean age of (28 ± 7) years served as the normal group. The duration of NSAA ranged from 6 months to 25 years.

Criteria for inclusion or exclusion into the study

Inclusion criteria: all subjects had primary NSAA. The diagnosis and decisions regarding curative effect were based on a Chinese publication: blood disease diagnosis and treatment standards.[9]

Exclusion criteria: the following subjects were excluded: pregnant or lactating women; those who could express themselves; patients with incomplete clinical data; those with insufficient cure (less than six months); patients who did not comply with treatment, who withdrew from the study, or who were involved in other clinical trials; subjects with severe and uncontrollable bleeding/infection; those who had other blood diseases or malignant diseases.[10]

Treatments

The experimental group underwent treatment with Busuishengxue granules. Each granule contains Shudihuang (*Radix Rehmanniae Preparata*), Shanyurou (*Fructus Corni*), Gouqizi (*Fructus Lycii*), Yinyanghuo (*Herba Epimedii*), Bajitian (*Radix Morindae Officinalis*), Lurong (*Cornu Cervi Pantotrichum*), Renshen (*Radix Ginseng*), Huangqi (*Radix Astragali*), Danshen (*Radix et Rhizoma Salviae Miltiorrhizae*), Jixueteng (*Caulis Spatholobi*), Baihuasheshecao (*Herba Hedyotis Diffusae*), and Zhuling (*Polyporus*). Busuishengxue granules were prepared by the Pharmaceutical Factory of the First Affiliated Hospital, Heilongjiang University of Chinese Medicine (lot number: 19970048; 15 g/bag). After being dissolved in water, one bag was taken three times daily *via* the oral route.[11]

The control group was treated with Zaizaoshengxue tablets (5 tablets on each occasion, three times daily). Zaizaoshengxue is a patented medicine in China approved by the State Food and Drug Administration (SFDA) for AA treatment. Each tablet comprises Tusizi (*Semen Cuscutae*), Hongshen (*Radix Ginseng Rubra*), Ejiao (*Colla Corii Asini*), Huangqi (*Radix Astragali Mongolici*), Danggui (*Radix Angelicae Sinensis*), Shudihuang (*Radix Rehmanniae Praeparata*), Heshouwu (*Radix Polygoni Multiflori* stir-fried), Yinyanghuo (*Herba Epimedii Brevicornus*), Huangjing (*Rhizoma Polygonati Sibirici*), Lurong (*Cornu Cervi Pantotrichum*), Xianhecao (*Herba Agrimoniae*) and Gouqizi (*Fructus Lycii*). It was produced by Liaoyuan Yadong Pharmaceutical Factory (Jilin Province, China; SFDA approval number, Z22025856; 0.35 g/tablet).

Both groups were treated for 3 months as one course. A follow-up of 12 months was conducted.

Measurements

Symptoms were scored according to guidelines for clinical research for TCMs.[12] The symptoms to be scored were: heart palpitations; dizziness; fatigue; pale face, mouth, lips and nails; night sweats; bleeding; cold peripheries; soreness and weakness of the waist and knees; fever; feverish palms and soles; thirst; dry stools; and loose stools. "0" denoted no symptoms, "1" slight symptoms, "2" medium symptoms, and "3" severe symptoms.

Determination of curative efficacy was based on a Chinese publication: blood disease diagnosis and treatment standards.[9] Four levels were used, as listed below.

(a) Basic recovery: symptoms of anemia and bleeding disappeared. Hemoglobin (HB) level for a male was ≥ 120 g/L and for a female was ≥100 g/L; WBCs ≥4×10^9/L;

platelets ≥80×10^9/L. No recurrence at >12-month follow-up.
(b) Remission: the symptoms of anemia and bleeding disappeared. HB level for a male was ≥120 g /L and for a female was ≥100 g/L; WBCs ≥3.5×10^9/L; platelets increased to some degree. The condition is stable or improving at 3-month follow-up.
(c) Significant improvement: the symptoms of anemia and bleeding improved significantly. Under a condition of no blood transfusion, the HB common increases to >30 g/L compared with 1 month before treatment and is maintained for ≥3 months. Those who fulfilled i-iii should not be transfused for 3 months.
(d) No effect: there was no significant improvement in symptoms and hemograms after treatment.[11]

Reagents and solutions

The medium for separating human lymphocytes was purchased from Tianjin Hao Yang Biological (Tianjin, China). Fluo-3-AM was from Signalway Antibody (College Park, MD, USA), as were monoclonal antibodies against p-JNK, p-p38 and p-ERK. β-actin and western blotting chemiluminescence solution were also from Signalway Antibody. Horseradish peroxidase (HRP)-labeled goat anti-rabbit immunoglobulin (Ig) G was from Beijing Zhongshan Golden Bridge Company (Beijing, China). Developing solution, fixer and X-OMAT BT film (5×7 in) were from Eastman Kodak (New York, NY, USA).

Detection methods

Flow cytometry can be used to detect $[Ca^{2+}]_i$ with lymphocyte separating medium. In this way, bone marrow mononuclear cells (BMMCs) were separated. Based on a Fluo-3-AM assay kit, Ca^{2+} in BMMCs was stained, and the separated BMMCs divided into tubes A and B. D-PBS (Dulbecco's Phosphate-Buffered Saline) was added to tube A, and Fluo-3AM to tube B. Both tubes were incubated in the dark for 1 h. Flow cytometry was then carried out on both tubes.
Western blotting was employed to detect the expression of enzymes and phosphorylation level. BMMCs were cultured in cell dishes. They were then rinsed with phosphate-buffered saline (PBS) and added to cell lysates. Cells were scraped and collected in 1.5 mL microcentrifuge tubes. This was followed by placement in a hot water bath for 5-10 min and centrifugation at 120 000 rpm for 10 min. The supernatant was removed to measure protein content using the Bradford method. Protein (50 μg) was taken from each sample to add to 2× sample buffer to conduct discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (separation gel, 100 g/L; spacer gel, 60 g/L). After electrophoresis, protein in the gel was electrotransferred to nitrocellulose membranes. Bovine serum albumin (10 g/L) was added drop-wise. Primary antibody (rabbit anti-ERK or rabbit anti-ERK antibody, working concentrations for both were 1∶1000 dilution) and secondary antibody (HRP-labelled goat-anti-rabbit IgG at 1∶3000 dilution) were added in order, and allowed to react for 1 h and 2 h, respectively. After washing membranes, detection was by ECL chemiluminescence (i.e., we developed an X-ray film for 2-20 min). The results after fixation were observed. The detection methods and monitoring of other indices were the same as described above.

Statistical analyses

Data are the mean ± standard deviation ($\bar{x} \pm s$). Group comparisons were tested by the Student's *t*-test. SPSS ver 13.0 (SPSS, Chicago, IL, USA) was employed to analyze data. $P<0.05$ was considered significant.

RESULTS

Curative efficacy of the experimental group and control group

The total efficacy of Busuishengxue granules and Zaizaoshengxue tablets was 80.0% and 53.3% respectively, and showed significant difference ($P<0.05$) (Table 1).

Symptom scores of the two groups before and after treatment

There was no significant difference between the two groups before treatment, but differences existed before and after treatment in both groups ($P<0.05$). There was a significant difference between the two groups after treatment ($P<0.05$)(Table 2).

Comparison of the peripheral blood of patients in the experimental group and control group

In peripheral blood, levels of WBC, HB and platelets in both groups after treatment increased compared with those before treatment ($P<0.05$). After treatment, platelet number was not significantly different ($P>0.05$), whereas WBCs and HB in the experimental group were significantly higher than those in the control group ($P<0.05$) (Table 3).

Table 1 Curative efficacy of the experimental group and control group [*n* (%)]

Group	*n*	Basic recovery	Remission	Significant improvement	No effect	Total efficacy
Experimental	30	0	14 (46.7)	10 (33.3)	0	24 (80.0)[a]
Control	30	0	4 (13.3)	12 (40.0)	14 (46.7)	16 (53.3)

Notes: the experimental group was treated with Busuishengxue granules (15 g/bag) three times daily. The control group was treated with Zaizaoshengxue tablets (5 tablets on each occasion, three times daily). Both groups were treated for 3 months as one course. A follow-up of 12 months was conducted. Comparison of the efficacy between the two groups is given by [a]$P<0.05$.

Table 2 Mean symptom scores of the experimental group and control group ($\bar{x}$ ±*s*)

Group	*n*	Treatment	Symptom score
Experimental	30	Before treatment	18±5
		After treatment	7±5[ab]
Control	30	Before treatment	18±5
		After treatment	11±5[a]

Notes: the experimental group was treated with Busuishengxue granules (15 g/bag) three times daily. The control group was treated with Zaizaoshengxue Tablets (5 tablets on each occasion, three times daily). Both groups were treated for 3 months as one course. A follow-up of 12 months was conducted. Group comparison before and after treatment is denoted by [a]*P*<0.05, and comparison between groups by [b]*P*<0.05.

Distribution of calcium ions and levels of bone marrow mononuclear cells in the experimental group and control group before and after treatment (Figure 1, Table 4)

The $[Ca^{2+}]_i$ in BMMCs of the experimental group and control group before treatment was higher than that of the normal group (*P*<0.0, Tables 4 and 5), whereas the $[Ca^{2+}]_i$ of the experimental group did not differ significantly from that of the control group (*P*>0.05). After treatment, the $[Ca^{2+}]_i$ in BMMCs decreased compared with that before treatment (*P*<0.05). The experimental group had better recovery than that of the control group (*P*<0.05).

MAPK/ERK pathway-related enzyme expression in bone marrow mononuclear cells

Expression of p-ERK1/2 protein in the experimental group and control group before treatment was significantly lower than that of the normal group (*P*<0.05, Table 5). There was no significant difference between the experimental group and control group before treatment (*P*>0.05). Expression of p- (extracellular regulated kinase) ERK1/2 in the experimental group and control group increased after treatment, differing significantly from that before treatment (*P*<0.05). There was a significant difference between the experimental group and control group after treatment (*P*<0.05), as well as with the normal group (*P*<0.05).

Expression of ERK1/2 protein in the experimental group and control group was lower than that of the normal group (*P*<0.01, Table 6), and the difference between the groups was significant (*P*>0.05). Expression after treatment in the experimental and control groups recovered to some degree, and the difference between groups was marked (*P*<0.05). The difference before and after treatment in the experimental group was significant (*P*<0.05), and was also significantly different from that of the normal group (*P*<0.01).

Expression of ERK2 protein in the experimental group and control group before treatment was significantly lower than that of the normal group (*P*<0.05, Table 7). ERK2 expression in the experimental group and control group after treatment increased significantly from

Table 3 Changes in the peripheral blood of patients in the experimental group and control group before and after treatment ($\bar{x}$ ±*s*)

Group	*n*	Treatment	WBC ($\times10^9$)	HB (g/L)	Platelets ($\times10^9$)
Experimental	30	Before treatment	2.4±0.4	59.2±17.2	21.2±6.5
		After treatment	3.5±1.0[ab]	87.1±18.5[ab]	42.3±7.7[ac]
Control	30	Before treatment	2.6±0.8	61.8±18.4	22.2±6.8
		After treatment	3.0±1.1[a]	72.9±19.6[a]	39.4±6.3[a]

Notes: the experimental group was treated with Busuishengxue granules (15 g/bag) three times daily. The control group was treated with Zaizaoshengxue tablets (5 tablets on each occasion, three times daily). Both groups were treated for 3 months as one course. A follow-up of 12 months was conducted. WBC: white blood cells; HB: Hemoglobin. Group comparison before and after treatment is given by [a]*P*<0.05, and comparison between groups after treatment by [b]*P*<0.05 and [c]*P*>0.05.

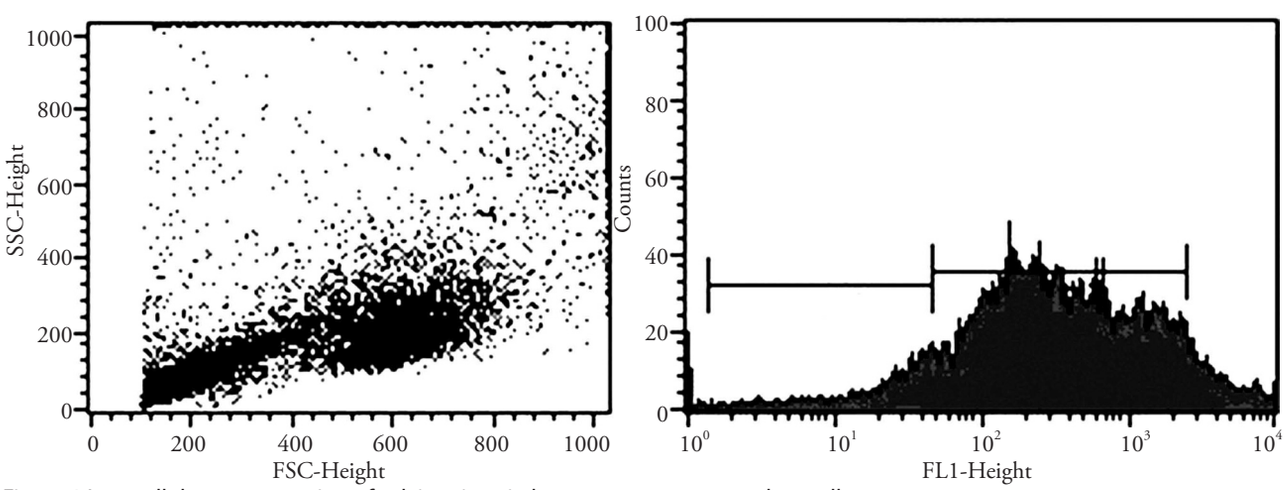

Figure 1 Intracellular concentration of calcium ions in bone marrow mononuclear cells
FSC: forward scatter; FL1: fluorescence channel first; SSC: side scatter.

Table 4 Distribution of calcium ions and levels of bone marrow mononuclear cells in the experimental group and control group before and after treatment ($\bar{x} \pm s$)

Group	*n*	$[Ca^{2+}]_i$	
		Before treatment	After treatment
Experimental	30	355±44[ab]	253±43[acd]
Control	30	354±45[a]	298±48[ac]
Normal	10	199±32	-

Notes: the experimental group was treated with Busuishengxue granules (15 g/bag) three times daily. The control group was treated with Zaizaoshengxue tablets (5 tablets on each occasion, three times daily). Both groups were treated for 3 months as one course. A follow-up of 12 months was conducted. Normal group were volunteers without any treatment. Comparison with the normal group is denoted by [a]*P*<0.05; before treatment, the inter-group comparison is denoted by [b]*P*>0.05; group comparison before and after treatment is denoted by [c]*P*<0.05; after treatment, the inter-group comparison is denoted by [d]*P*<0.05.

Table 5 Expression of p-ERK1/2 protein in the experimental group and control group before and after treatment ($\bar{x} \pm s$)

Group	*n*	p-ERK1/2 (grayscale analyses)	
		Before treatment	After treatment
Experimental	30	0.93±0.19[ab]	1.24±0.21[acd]
Control	30	0.89±0.25[a]	1.05±0.25[ac]
Normal	10	1.59±0.17	-

Notes: the experimental group was treated with Busuishengxue granules (15 g/bag) three times daily. The control group was treated with Zaizaoshengxue tablets (5 tablets on each occasion, three times daily). Both groups were treated for 3 months as one course. A follow-up of 12 months was conducted. Normal group were volunteers without any treatment. p-ERK1/2: p-extracellular signal regulated kinase 1/2. Comparison with the normal group is denoted by [a]*P*<0.05; comparison between groups before treatment is denoted by [b]*P*>0.05; group comparison before and after treatment is denoted by [c]*P*<0.05; comparison between groups after treatment is denoted by [d]*P*<0.05.

Table 6 Expression of ERK1/2 protein in bone marrow mononuclear cells of the experimental group and control group before and after treatment ($\bar{x} \pm s$)

Group	*n*	ERK1 (grayscale analyses)	
		Before treatment	After treatment
Experimental	30	5.96±0.28[ab]	7.95±0.32[cdf]
Control	30	5.46±0.31[a]	6.94±0.37[ae]
Normal	10	9.50±0.13	-

Notes: the experimental group was treated with Busuishengxue granules (15 g/bag) three times daily. The control group was treated with Zaizaoshengxue tablets (5 tablets on each occasion, three times daily). Both groups were treated for 3 months as one course. A follow-up of 12 months was conducted. Normal group were volunteers without any treatment. ERK1: extracellular signal regulated kinase 1. Compared with the normal group is denoted by [c]*P*<0.05 and [a]*P*<0.01; comparison before treatment is denoted by [d]*P*<0.05 and [c]*P*>0.05; comparison with the control group is denoted by [f]*P*<0.05 and [b]*P*>0.05.

Table 7 Expression of ERK2 protein in bone marrow mononuclear cells of the experimental group and control group before and after treatment ($\bar{x} \pm s$)

Group	*n*	ERK2 (grayscale analyses)	
		Before treatment	After treatment
Experimental	30	3.68±0.31[a]	5.84±0.32[abc]
Control	30	3.71±0.28[a]	4.21±0.31[ab]
Normal	10	6.62±0.13	-

Notes: the experimental group was treated with Busuishengxue granules (15 g/bag) three times daily. The control group was treated with Zaizaoshengxue tablets (5 tablets on each occasion, three times daily). Both groups were treated for 3 months as one course. A follow-up of 12 months was conducted. Normal group were volunteers without any treatment. ERK 2: extracellular regulated kinase 2. Comparison with the normal group is denoted by [a]*P*<0.05; comparison with before treatment is denoted by [b]*P*<0.05; comparison with the control group is denoted by [c]*P*<0.05.

that before treatment (*P*<0.05). There was a significant difference between the experimental group and control group after treatment (*P*<0.05), as well as compared with the normal group (*P*<0.05).

Expression of p-JNK protein in BMMCs of the experimental group and control group before treatment was significantly higher than that of the normal group (*P*<0.05, Table 8). After treatment, expression of p-JNK protein in the experimental group and control group decreased, and differed significantly to that before treatment (*P*<0.05). There was an obvious difference between the experimental group and control group after treatment (*P*<0.05), and a significant difference was noted compared with the normal group (*P*<0.05).

Table 8 Expression of p-JNK protein in bone marrow mononuclear cells of the experimental group and control group before and after treatment ($\bar{x} \pm s$)

Group	*n*	p-JNK (grayscale analyses)	
		Before treatment	After treatment
Experimental	30	1.27±0.22[a]	0.98±0.22[abc]
Control	30	1.28±0.21[a]	1.12±0.24[ab]
Normal	10	0.89±0.20	-

Notes: the experimental group was treated with Busuishengxue granules (15 g/bag) three times daily. The control group was treated with Zaizaoshengxue tablets (5 tablets on each occasion, three times daily). Both groups were treated for 3 months as one course. A follow-up of 12 months was conducted. Normal group were volunteers without any treatment. p-JNK: p-c-Jun N-terminal kinases. Comparison with the normal group is denoted by [a]*P*<0.05 and [b]*P*>0.05; group comparison before and after treatment is denoted by [c]*P*<0.05.

Expression of p-p38 protein in BMMCs of the experimental group and control group before treatment was significantly higher than that of the normal group (*P*<0.05, Table 9). After treatment, expression of p-p38 protein in the experimental group and control group decreased, and differed significantly to that before treatment (*P*<0.05). There was no remarkable difference be-

Table 9 Expression of p-p38 protein in bone marrow mononuclear cells of the experimental group and control group before and after treatment ($\bar{x}$ ±s)

Group	*n*	p-p38 (grayscale analyses)	
		Before treatment	After treatment
Experimental	30	2.32±0.15[a]	1.93±0.16[bcd]
Control	30	2.35±0.22[a]	2.19±0.23[ac]
Normal	10	1.88±0.13	-

Notes: the experimental group was treated with Busuishengxue granules (15 g/bag) three times daily. The control group was treated with Zaizaoshengxue tablets (5 tablets on each occasion, three times daily). Both groups were treated for 3 months as one course. A follow-up of 12 months was conducted. Normal group were volunteers without any treatment. Comparison with the normal group is denoted by [a]P<0.05 and [b]P>0.05; group comparison before and after treatment is denoted by [c]P<0.05; comparison with the control group after treatment is denoted by [d]P<0.05.

tween the experimental group and normal group after treatment (P>0.05), but there was a significant difference between the experimental group and control group after treatment (P<0.05).

Expression of p-ERK5 protein in the experimental group and control group was significantly lower than that of the normal group before treatment (P<0.05, Table 10). Expression of p-ERK5 protein in the experimental group and control group increased after treatment, and was significantly different from that before treatment (P<0.05). There was an obvious difference between the experimental group and control group after treatment (P<0.05), and a significant difference was noted compared with the normal group (P<0.05).

Table 10 Expression of p-ERK5 protein in bone marrow mononuclear cells of the experimental group and control group before and after treatment ($\bar{x}$ ±s)

Group	*n*	p-ERK5 (grayscale analyses)	
		Before treatment	After treatment
Experimental	30	0.65±0.25[a]	1.57±0.30[abc]
Control	30	0.63±0.21[a]	1.25±0.36[ab]
Normal	10	1.87±0.56	-

Notes: the experimental group was treated with Busuishengxue granules (15 g/bag) three times daily. The control group was treated with Zaizaoshengxue tablets (5 tablets on each occasion, three times daily). Both groups were treated for 3 months as one course. A follow-up of 12 months was conducted. Normal group were volunteers without any treatment. p-ERK5: p-extracellular signal regulated kinase 5. Comparison with the normal group is denoted by [a]P<0.05; comparison in the group before and after treatment is denoted by [b]P<0.05; comparison with the control group after treatment is denoted by [c]P<0.05.

DISCUSSION

When evaluating clinical efficacy, we found that the total efficacy of Busuishengxue granules and Zaizaoshengxue tablets was 80.0% and 53.3% (P<0.05) respectively. Busuishengxue granules had a better effect on symptom scores and increasing the number of leukocytes and HB levels in peripheral blood than Zaizaoshengxue tablets, whereas they differed slightly with regard to increasing the number of platelets.

Adhesion molecules can be divided by structure into the integrin family, Ig superfamily, selectin, cadherin, glycoprotein, CD44 and sialomucin. There are three mechanisms of cell adhesion. The first is mutual recognition and binding of the same cell adhesion molecule on two adjacent cell surfaces. The second is mutual recognition and binding of different cell adhesion molecules on two adjacent cell surfaces. The third type is mutual recognition and binding of the same cell adhesion molecule on two adjacent cell surfaces by means of extracellular linker molecules.

Integrins deliver membrane molecules that transduce signals in a special way. Hence, several cell phenotypes can be altered by integrins, such as the growth, differentiation, migration, invasion and apoptosis of cells.[13,14] By combination with different ligands, various signal transduction pathways can be activated by an integrin. The major signal transduction pathways are the tyrosine kinase-dependent pathways: the focal adhesion kinase (FAK) pathway and Shc pathway. There may be many types of signal transduction pathways after integrins activate the FAK pathway. The FAK-Ras-MAPK pathway has been studied quite comprehensively and involves two aspects. First, after the combination of FAK and Src, Crk-associated substrate (Cas) and paxillin can be phosphorylated. After phosphorylation of their butyric-acid moieties, they can adjust the cytoskeleton and produce domain proteins containing Src homology-2 (SH2), such as the binding site of Crk (CT2102-regulated kinase). Thus, the Ras/MAPK pathway can be activated *via* Crk. Second, Tyr925 phosphorylation of FAKs is caused by a combination of FAK and Src, thereby providing the adapter protein Grb2 with a binding site; the Src homology-3 (SH3) of Grb2 can be linked with son of sevenless (SOS). The SOS protein is a guanine nucleotide exchange factor that connects with the receptor phosphorylated in tyrosine residues *via* connexin Grb2. Therefore, through Grb2, FAKs can also activate the Ras/MAPK pathway. The activated MAPK can activate many transcription factors and mediate the activity expressed by the FAK regulator gene.[13-15]

Our experimental results showed abnormal expression of the enzymes of the signal transduction pathway related to the BMMCs of NSAA patients. This expression was significantly different from that of normal subjects. Our data suggested that NSSA patients had an abnormal Ras/MAPK pathway. Before treatment, expression of p-ERK1, p-ERK2, p-ERK1/2 and p-ERK5 was lower than that of normal controls, whereas expression of JNK and p-p38 as well as $[Ca^{2+}]_i$ was higher than those of normal controls. These data suggested abnormal adhesion signals in the blood cells of NSAA patients.

After treatment, expression of p-ERK1, p-ERK2, p-ERK1/2 and p-ERK5 of NSAA patients increased to different degrees. Expression of JNK and p-p38 was down-regulated and $[Ca^{2+}]_i$ reduced to different degrees but, after treatment, except for p-p38 (P<0.05), the indices in the experimental group were still significantly different from those of the normal group.

We inferred from previous studies that the abnormal expression of integrins from the VLA subfamily may result from the abnormal expression of enzymes of the intracellular signal pathway mediated by them. That is, abnormal intracellular signaling pathways may lead to abnormal adhesion molecules being expressed on the surface of BMMCs as well as expression of abnormal ligands. These events could affect the normal "homing" of hematopoietic cells in the BM, and affect their differentiation, proliferation and apoptosis, thereby leading to NSAA.

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